The development and characterization of glial-specific cell lines in *Drosophila*

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Molecular Genetics in the undergraduate colleges of The Ohio State University

By

Molly Josifov

The Ohio State University

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Project Advisor: Dr. Amanda Simcox, Department of Molecular Genetics

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ABSTRACT

Glioblastoma multiforme (GBM) is one of the most common and aggressive human brain tumors, accounting for 52% of all primary brain tumors. The median survival for a person diagnosed with GBM is 15 months. GBM originates in glial cells, cells that provide support and protection to neurons in the nervous system. The current treatments for glioblastoma include surgery and chemotherapy, but prolonged remission is rare with the median time for tumor recurrence being about 6.9 months. GBM is currently being studied in human cells and animal models, including the fruit fly *Drosophila melanogaster*. Fly glioblastoma models have been developed, but there was no equivalent cell culture model of the glioblastoma in flies. I aimed to develop a fly-cell model of glioblastoma. I am generating cell culture line of glial cells by expressing an oncogene, Ras^{V12}, which is known to be involved in GBM, and by repression of tumor suppressors, such as *brat*. The human homolog of *brat*, TRIM3, has been implicated in GBM. To date, I have developed one cell line. To determine if the cell line I generated is of glial-cell type, I analyzed the cells using a glial specific cell marker, confirming that the cells were indeed glial. The glial cell line that has been developed has the potential to be useful in understanding the basic mechanisms of the disease and for testing therapies.

ACKNOWLEDGMENTS

I would like to thank the members of the Simcox Lab for mentoring and assisting me in my research over the last three years and for advice in the editing of this thesis (Dr. Sathiya Manivannan, Dr. Delphine Fagegaltier, Dr. Uyen Tram, Ashley Heinaman, Geeta Palsule, Shiva Raghuvanshi). Special thanks to Dr. Amanda Simcox for allowing me to be part of her lab for the past three years and for taking time to mentor me into a research scientist. I would like to thank Dr. Anita Hopper for serving on my committee. Finally, thank you to the College of Arts and Sciences for the Undergraduate Research Scholarship and to Pelotonia for the Undergraduate Research Fellowship to support my work.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most invasive type of glial tumor in which malignant cells can rapidly spread to nearby brain tissue. GBM most commonly originates in the cerebral hemispheres of the brain and complete removal of the tumor without damaging vital normal brain tissue is impossible due to the dispersal of the malignant cells. Because of the particularly infiltrative and aggressive properties of GBM, current treatments including surgery and chemotherapy often provide a higher quality of life for the diagnosed, but do not result in the complete removal of cancerous tissue. GBM typically results in death 15 months after the initial diagnosis (AANS - Glioblastoma Multiforme n.d.). According to the National Cancer Institute, of the 22,850 American men and women diagnosed with brain or other nervous system cancer in 2015, 15,320 people have already died (The RAS Problem - National Cancer Institute n.d.). The 5-year survival rate for GBM is 4% (Tumor Types- National Brain Tumor Society n.d.).

Drosophila melanogaster is a highly-studied model organism with well-defined genetics. Although simpler, the *Drosophila* central nervous system shares many similarities with that of the mammalian system. There are neurons and glia. Both the *Drosophila* glial blood-brain barrier and the mammalian endothelial blood-brain barrier protect the nervous system from pathogens and regulate the concentrations of solutes and metabolites (Limmer et al. 2014). In addition, the organization of the brain is similar. (McGurk, Berson, and Bonini 2015). In both mammals and *Drosophila,* glial cells are non-neuronal cells responsible for supporting the nervous system, including providing nutrients to the neurons and maintaining extracellular ion balance. There are three main classes of mammalian glial cells in the central nervous system, each with distinct morphologies and roles: oligodendrocytes, microglia, and astrocytes.

Likewise, there are three main classes of neuron-associated *Drosophila* glial cells: astrocytes, cortex glia, and ensheathing glia. Together, these three classes share noteworthy molecular and functional characteristics with mammalian glial cells (Freeman 2015). Because of these similarities, *Drosophila* is an effective model for studying GBM. In addition, the fruit fly genome (1.2×10^8 base pairs) is simpler than that of the human genome (3.3×10^9 base pairs), with about 6,000-11,000 fewer protein-coding genes. In humans, many of these additional genes perform overlapping functions. The less redundant *Drosophila* genome is therefore useful for loss of function studies in which phenotypes might be seen that are masked in the more complex genomes of other animals.

Drosophila has been an effective *in vivo* model of gliomas. In mammals, gliomas commonly occur when the EGFR and P13K pathways are both activated (Furnari et al. 2007; Maher et al. 2001). In flies, the activation of these pathways in embryonic glial cells resulted in over-proliferating glial cells. Upon injection into adults, the cells invaded and grew, forming tumors through the activation of a network of oncogenic genes (Read et al. 2009).

Drosophila is an effective model for studying cancer; the roles of many tumor suppressor and oncogene pathways in brain cancer have been studied using a fly model. For example, experiments performed in *Drosophila* provided some of the first evidence of the regulation of Ras pathway signaling by the NF1 gene encoding neurofibromin, a protein associated with the Neurofibromatosis Type 1 brain cancer in children, characterized by the unchecked growth of tissues along nerve cells (Read et al. 2009; Rudrapatna, Cagan, and Das 2012).

Drosophila has been used as a whole-animal model for GBM (Read et al. 2009), but there is no corresponding fly cell culture model of glioblastoma. A fly cell culture model of GBM would provide an alternative environment as a useful complement to an *in vivo* model, in

which RNAi screens related to GBM have already been conducted (Read et al. 2013). A cell line provides a large amount of homogenous cells that can be more easily manipulated (Cherbas and Gong 2014). RNA interference (RNAi) in cell culture is a cheap, effective, and simple way to silence genes (Caplen et al. 2000; Clemens et al. 2000). Using a fly-cell culture model of GBM in complement to a whole-animal model could help us find novel factors blocking the progression of GBM and could lead to the development of new therapies.

Fly cell lines have some advantages over mammalian cell lines. They remain alive at room temperature and atmospheric levels of CO_2, making the handling and imaging of *Drosophila* cell lines much easier (Baum and Cherbas 2008).

I have developed a glial-specific *Drosophila* cell line using the *Gal4/UAS* system, a system in which a specific subset of cells (glial cells) can be induced to express an oncogene, Ras^{V12}. Ras^{V12} is a protein in the Epidermal Growth Factor Receptor Pathway with a role in 30% of human cancers, including GBM. The *Gal4* driver that I used is a well-known glial-specific driver, *repo-Gal4* (Yuasa et al. 2003). Shown in Figure 1, the *repo* protein is expressed in the glial nuclei of developing *Drosophila*.

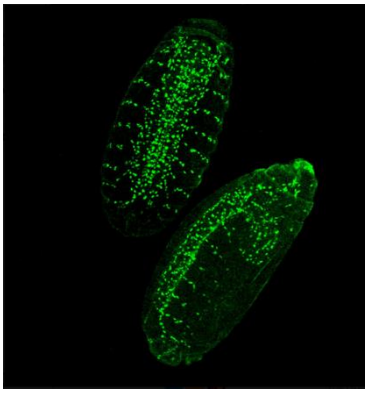


Figure 1: anti-*Repo* antibody staining of embryo. *Repo* is expressed in the glial cells of the nervous system. Photo courtesy of Karla Daniels.

MATERIALS AND METHODS

Fly Stocks

The following gene alleles and transgenes were used: *repo-Gal4, UAS-Ras^{V12}; UAS-GFP, UAS-Ras^{V12}, P[attP.w+.attP]JB89B/TM3* (Bateman, Lee, and Wu 2006), *UAS-P35, UAS-Ras^{V12}, UAS-wts^{dsRNA}; UAS-Ras^{V12} ; UAS-wts^{dsRNA}; tubG80TS; UAS-Ras^{V12}*.

Gal4/UAS System

I utilized the *Gal4/UAS* system to generate glial cells that express *Ras^{V12}*. To establish the lines, I crossed two transgenic fly lines: the first line expressing the *Gal4* gene in glial cells and the second line carrying the *Upstream Activating Sequence* (*UAS*), to which *Gal4* binds, upstream of *Ras^{V12}* or other genes of interest. The *Gal4* is expressed only in the glial cells, activating transcription of either *Ras^{V12}* or these genes and leading to the proliferation of glial cells in culture (Brand and Perrimon 1993; Simcox et al. 2008).

Primary culture

Embryos were collected every 12 hours at room temperature, rinsed from a petri dish and collected in a sieve. The embryos were washed using TXN (0.7% NaCl, 0.02% Triton X-100), then 50% bleach in water for 3–5 minutes to remove the chorion and surface sterilize the embryos. The embryos were washed 6 times with TXN and transferred to a homogenizer (Wheaton 5 ml). The embryos were rinsed once in sterile water and once in 2 ml medium (Schneider's medium, supplemented with 10% heat-inactivated fetal bovine serum, and 1/100 dilution of streptomycin penicillin liquid, Invitrogen) and then homogenized in 3 ml medium . The cells were pelleted by centrifugation and rinsed with three changes of medium. The cells were plated in 12.5 cm^2 T-flasks and grown at various termperatures (18-25 ºC). To maintain the

primary cultures, the medium (Schneider's Insect Medium) was changed every 10 days (Simcox et al. 2008).

Passaging

Confluent cultures were trypsinized and diluted 1/2–1/4. Early passages were often difficult to establish and slow to grow to confluence, so were not diluted. The parent culture was maintained (with fresh medium) and used to establish multiple first passage cultures before one line showed successful continued growth (Simcox et al. 2008).

Immunohistochemistry

Cells were grown in 6-well plates for antibody staining. Cells were washed once in 1× PBS and fixed for 20 minutes in 4% paraformaldheyde in PBS. Cells were washed in PBS and permeablized with PBS+0.2% Triton X-100 (PBTX). Cells were washed in PBS and blocked in PBS with 5% Normal Goat Serum (NGS) for 1 hour and incubated with primary antibody and 5% NGS, overnight at 4°C. Cells were washed PBS and secondary antibodies (1:100) were added and incubated for 30 mins-1 hour at room temperature. Cells were washed in PBS and mounted using VectaShield (Vector Laboratories). Images were captured using a compound fluorescence microscope (Simcox et al. 2008). The following antibodies were used: 8D12 anti-*repo*, 22C10, 1D4 anti-*Fasciclin* II (Hybridoma), anti-*HRP*.

Karyotyping

Cells at about 50% confluence were incubated with a final concentration of 0.01µg/ml N-desacetyl-N-methylocolchicine (KaryoMAX, Gibco) for 3 hours. Cells were harvested with trypsin, washed in Phosphate buffered saline (PBS) and resuspended in 5 ml 0.075 M KCl and 1.3 mM sodium acetate (hypotonic solution) for 30 minutes. Four drops of fix (3:1 methanol:glacial acetic acid) were added and the cells were centrifuged, resuspended in

fix and incubated for 10 minutes. Cells were centrifuged, resuspended in a small quantity of fix and dropped onto clean slides. Slides were mounted in VECTASHIELD with DAPI (Vector Laboratories) and approximately 50 mitotic spreads were scored (Manivannan et al. 2015).

RESULTS AND DISCUSSION

Primary cultures under the control of repo-Gal4 expressing Ras^{V12} and GFP

The *Gal4* driver that I used is a well-known glial-specific driver, *repo-Gal4*. *Repo*, short for *reversed polarity*, is a homeodomain transcription factor expressed in glial cells and important in glial cell differentiation (Yuasa et al. 2003). To generate a glial cell line, I crossed *repo-Gal4* to *UAS Ras^{V12}*, *UAS-GFP* and the progression of a primary culture from the initial stages to confluence is shown in Figure 2. Primary cultures contain many cells and cell types initially and are confluent at around 20-30 days. I was unable to establish continuous cell lines using the reporter *UAS-Ras^{V12}*,*UAS-GFP*.

repo-Gal4; UAS-Ras^{V12}; RMCE

repo-Gal4 was used to drive the expression of *UAS-Ras^{V12}*. The fly stock and cells also had an attP flanked genomic cassette, a target for Recombination Mediated Cassette Exchange, which can be used to later insert transgenes of interest into the cells (Manivannan et al. 2015). Eight primary cultures were made and seven were passaged at least once. Just like the *repo-Gal4; UAS-Ras^{V12}, UAS-GFP* cells, no cell lines were generated from this cross, leading to our belief that *Ras^{V12}* expression alone would not lead to a glial-specific cell line (Figure 3). In other animal models, such as in the mouse, expression of *Ras^{V12}* in cell culture leads to premature senescence unless another oncogene is involved or a tumor suppressor is inactivated (Serrano et al. 1997).

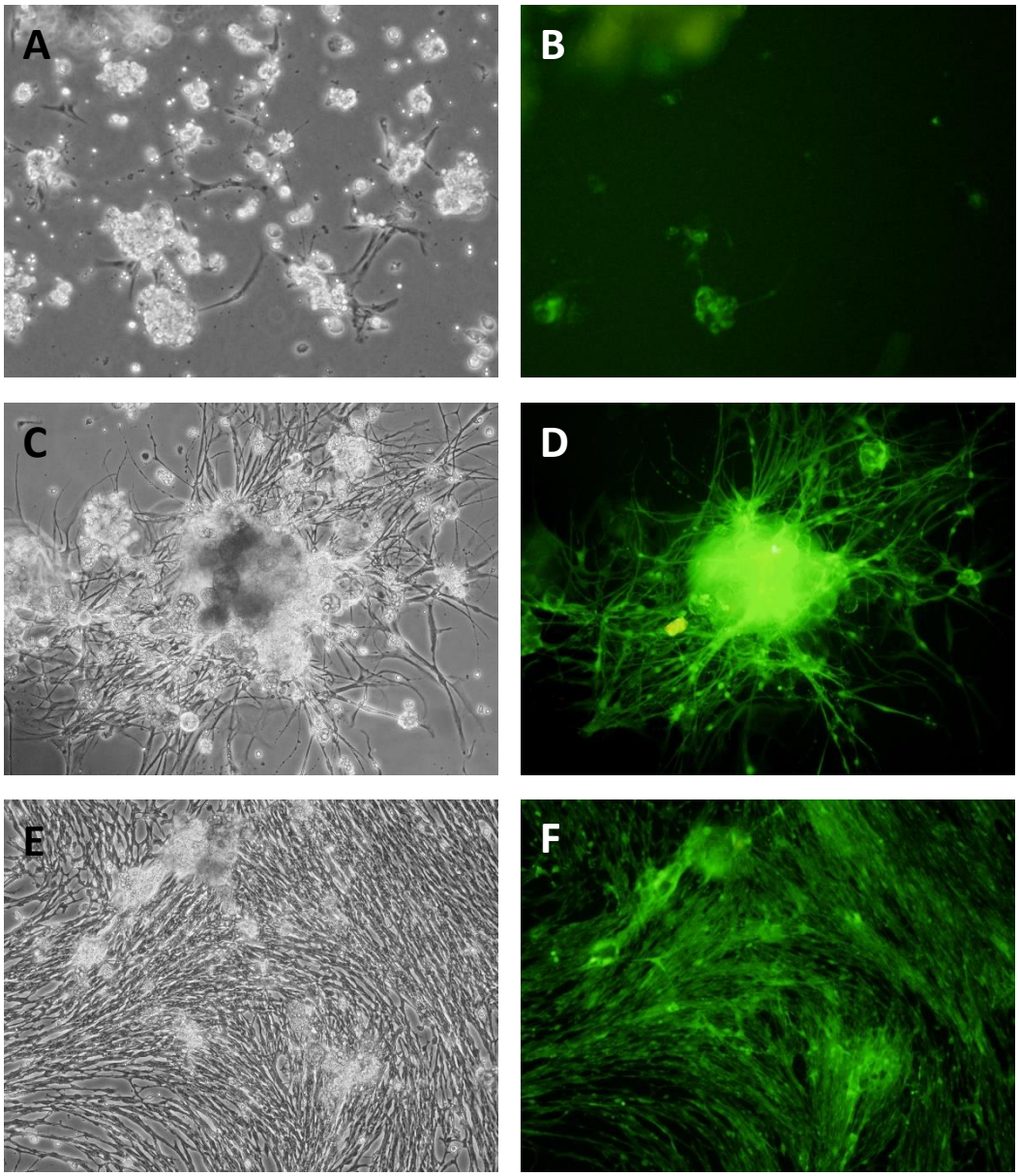


Figure 2: *repo-Gal4 ; UAS-Ras*^{V12}*,UAS-GFP* primary culture. GFP expression present in cells expressing *repo*, glial cells.. A,B: Phase and GFP image of a primary culture at day 2. Different cell types are present in the primary culture. Cells marked by GFP are glial. C,D: Phase and GFP images of a primary culture at day 12. There are proliferating patches in culture. E,F: Phase and GFP images of a primary culture at day 14. The cell culture flask is confluent with cells expressing GFP, glial cells.

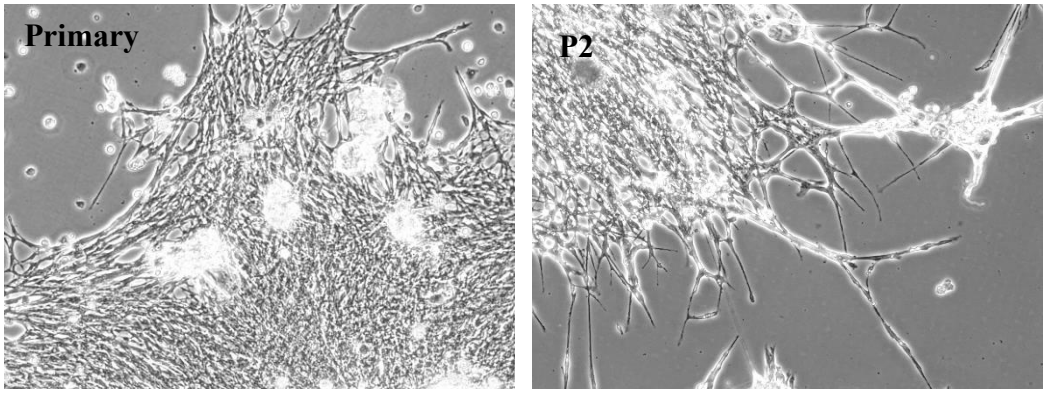


Figure 3: *repo-Gal4; UAS-Ras^{V12}; RMCE* cells. The primary culture became confluent and was passaged on day 19. Cells in later passages are unhealthy and do not reach confluence before dying and lifting off the plate.

Establishment of a glial-specific cell line

Based on the expression of *UAS-Ras^{V12} ,UAS-GFP*, we decided that *repo-Gal4* was a good pan-glial driver, but we would need to utilize a different responder line to generate the cell line, we could not utilize expression of *Ras^{V12}* alone. We crossed the *repo-Gal4* stock with a variety of other responder lines. Included in this list is *UAS- wts*^{dsRNA} , *UAS-Ras^{V12} ;UAS-wts* ^{dsRNA}, *UAS-P35, UAS-Ras^{V12}*, and tub-*Gal80; UAS-Ras^{V12}*.

repo-Gal4; UAS-wts^{dsRNA}

Repo-Gal4 was used to drive the expression of *wts*^{dsRNA} in culture. *wts* is a known *Drosophila* tumor suppressor (Justice et al. 1995), so knocking down the expression of *wts* in glial cells could lead to the proliferation of glial cells in culture. Out of five *repo-Gal4 ; UAS-wts*^{dsRNA} primaries, two primary cultures led to lines. These lines, however, stained negative for *repo*, indicating that they were not glial in nature (Figure 4).

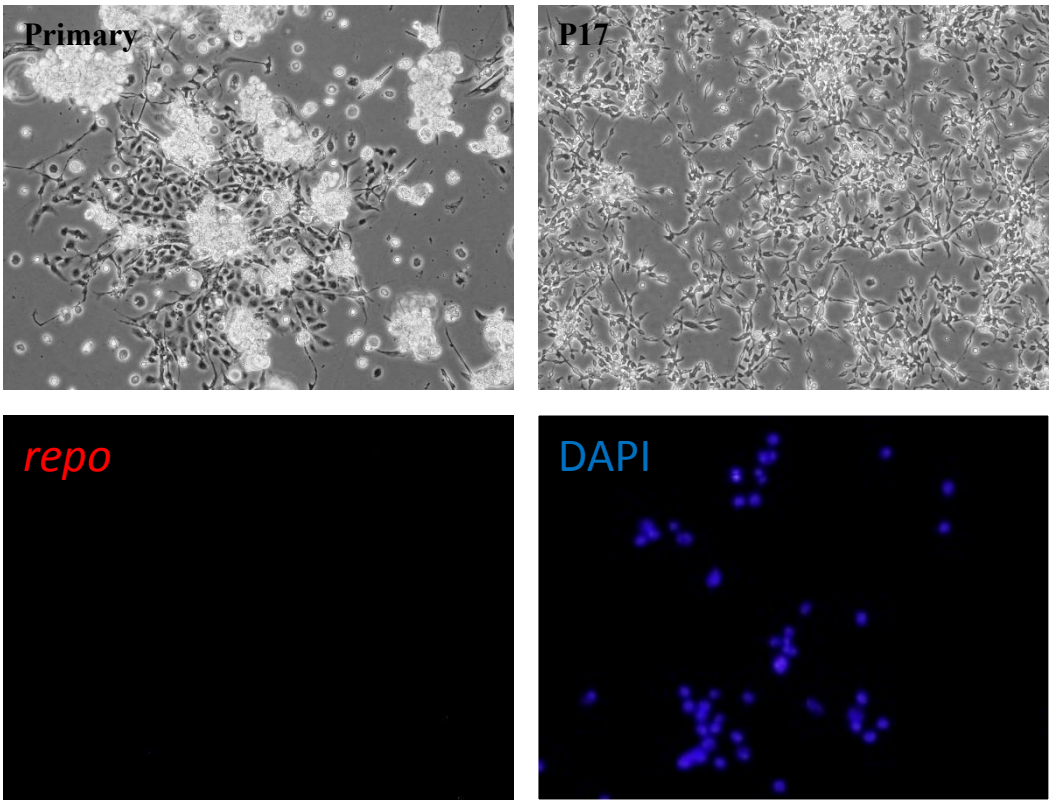


Figure 4: *repo-Gal4; UAS-wts* ^{dsRNA} cell line. A cell line was developed using *repo-Gal4* and *UAS-wts* ^{dsRNA}. Top left: Primary culture at 26 days. Top right: Passage 17 of the cell line. Bottom left: Cell line is stained with anti-repo antibody and the result is negative. Bottom right: DAPI staining illustrates the presence of cells in the field of view.

repo-Gal4; UAS-Ras^{V12}; UAS-wts^{dsRNA}

I next tested *UAS-Ras^{V12}* in combination with *UAS-wts* ^{dsRNA} to test whether they would increase the likelihood of glial cell proliferation in culture. None of the five primaries of *repo-Gal4; UAS-Ras^{V12}* ; *UAS-wts*^{dsRNA}led to cell lines; the cells in the primary culture grew very quickly and appeared unhealthy in later passages, leading to death (Figure 5).

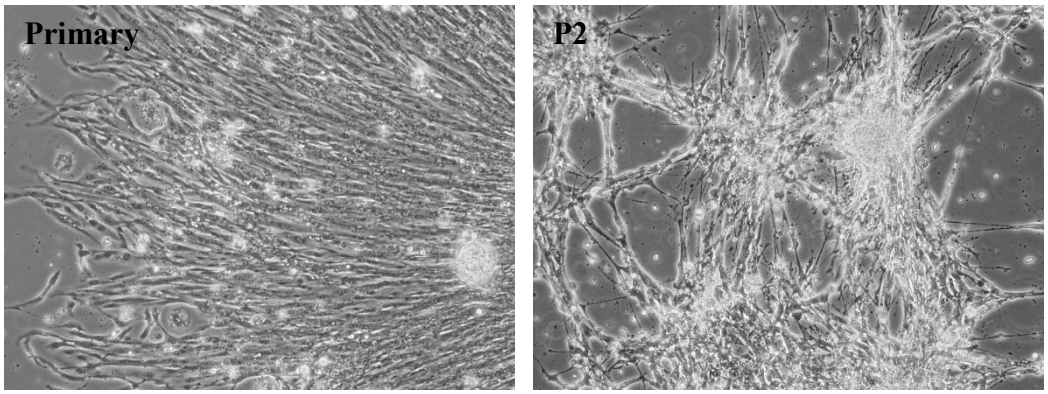


Figure 5: *repo-Gal4; UAS-Ras^{V12}; UAS-wts^{dsRNA}* cells. Cells were confluent and passaged after 13 days. left: primary culture. right: cells were dying and lifting off the plate in passage 2.

repo-Gal4; UAS-P35, UAS Ras^{V12}

Next, to determine if blocking cell death would generate glial-specific cell lines, I crossed *repo-Gal4* to *UAS-P35, UAS-Ras^{V12}*. *P35* is a protein that blocks cell death, including cells in developing embryos (Hay, Wolff, and Rubin 1994). From six primaries, no cell lines were developed. On average, at passage 3, the cells began to look unhealthy, lifting off of the flask surface before reaching confluency (Figure 6). In addition, *UAS-P35, UAS Ras^{V12}* flies with an RMCE site were crossed with *repo-Gal4* and the results were similar to the results of the cross with no RMCE site, as expected, because the RMCE site is a "neutral" element for future transgene insertion. A summary of the primary cultures resulting from the cross is in Table 1.

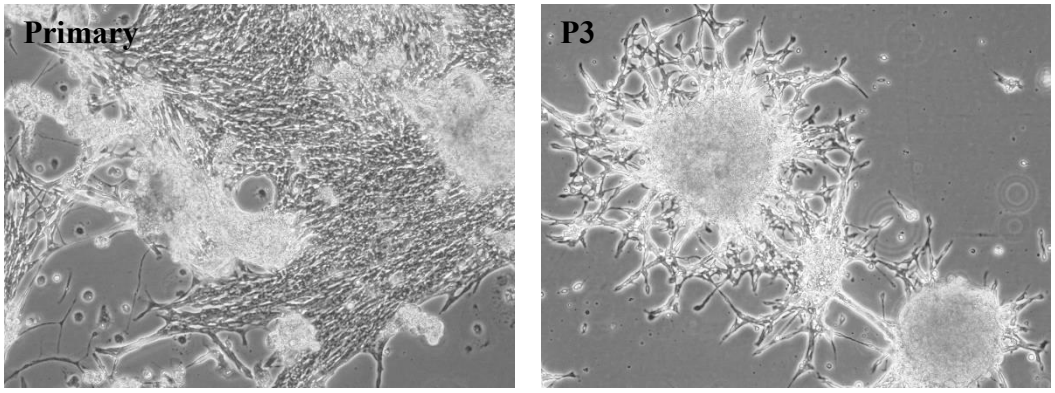


Figure 6: *repo-Gal4; UAS-P35, UAS-Ras^{V12}* cells. Cells were confluent and passaged after 22 days. left: primary culture. right: cells were unhealthy and dying in passage 3.

tub-Gal80ts; repo-Gal4; UAS-Ras^{V12}

Reducing *Ras^{V12}* expression proved to be key for continued growth. I successfully derived a cell line by controlling the level of expression of *Ras^{V12}* using the temperature sensitive *Gal4* inhibitor, *Gal80* (Suster et al. 2004). At lower temperatures, *Gal80* reduces the expression of *Ras^{V12}* by repressing *Gal4* in some cells (Suster et al. 2004). Primaries of *tub-Gal80ts; repo-Gal4; UAS-Ras^{V12}* were plated. From six primary cultures, one line was developed termed RepoG80-6. The passages of this line were grown at three different temperatures: 25°C, 22°C, and 18°C and in different conditions of medium: Schneider's Insect Media, 10%FBS (Fetal Bovine Serum) and Penicillin-Streptomycin with and without 10-20% pyruvate and Schneider's Insect Media, 10%FBS. The line grew the most successfully at 18°C in Schneider's Insect Media supplemented only with FBS, when *Ras^{V12}* expression was at its lowest. As seen in Figure 7, RepoG806 has been passaged over ten times, indicating it is a cell line.

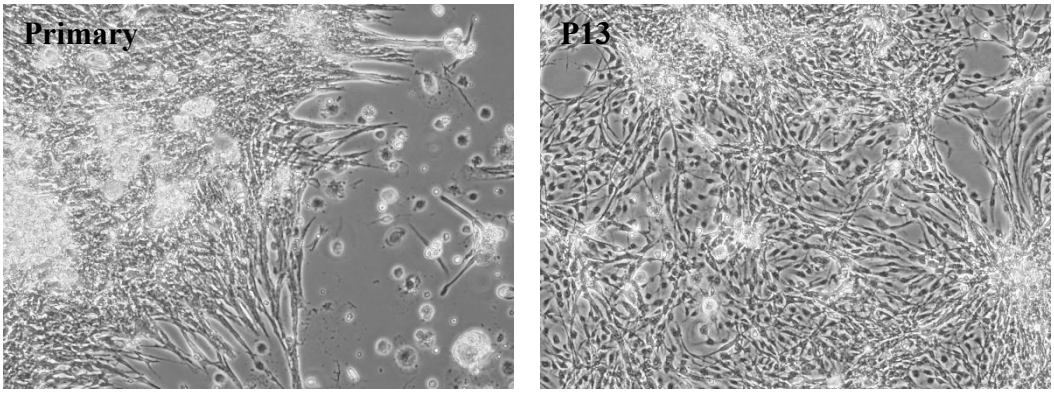
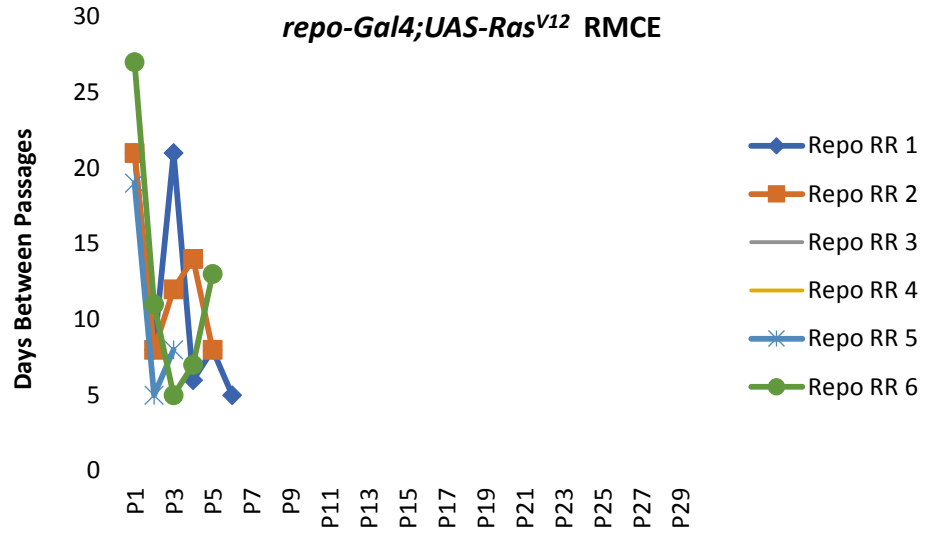


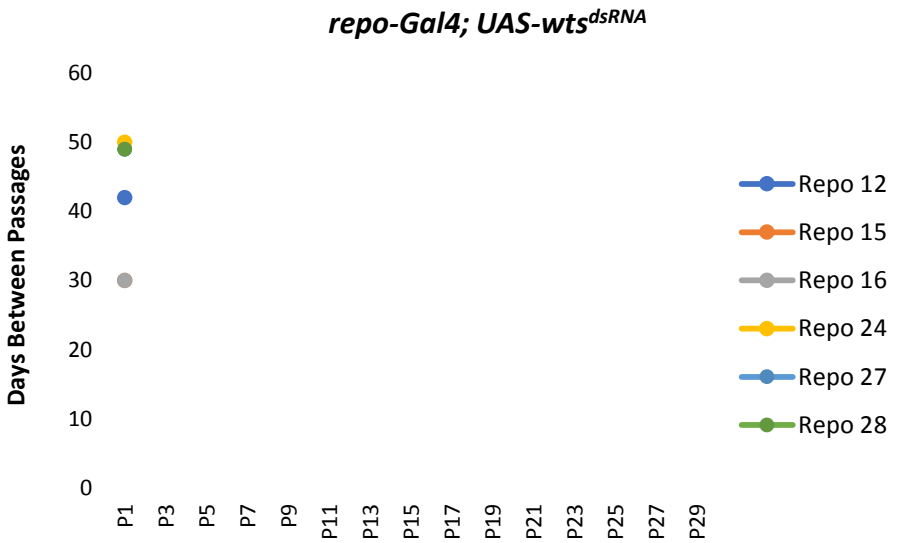
Figure 7: *tub-Gal80TS; repo-Gal4; UAS-Ras^{V12}* cells. Cells were confluent and passaged after 26 days. left: primary culture. right: cells as a line at passage 13.

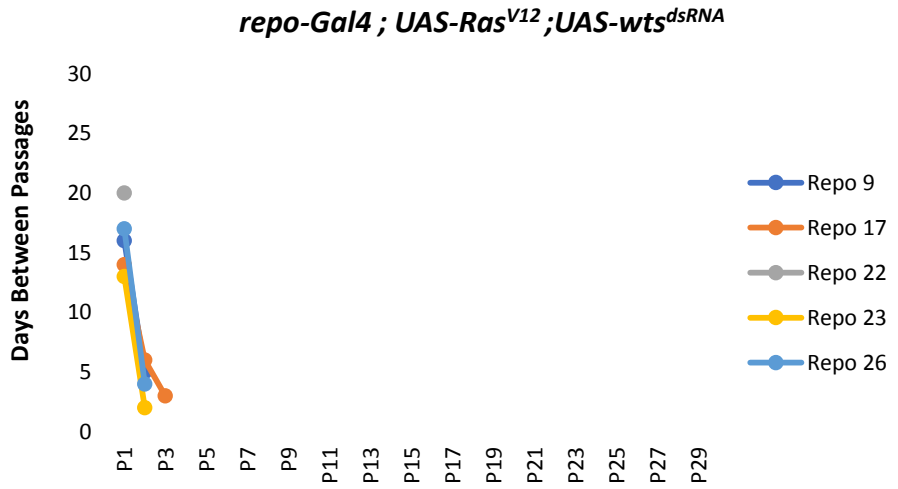
A summary of reporter lines crossed with *repo-Gal4*, number of primaries, and cell lines generated is shown in Table 1. Figure 8 shows growth charts for each set of primaries, organized by responder line.

repo-Gal4 > (glial-specific driver)	**UAS-Ras^{V12} (+/- attP*)**	**UAS-wts dsRNA**	**UAS-Ras^{V12}; UAS-wts dsRNA**	**UAS-P35, UAS-Ras^{V12} (+/- attP*)**	**Tub-Gal80TS; UAS-Ras^{V12}**
Number of primary cultures	6	5	5	10	6
Days to confluence	22	42	16	21	34
Number of cell lines developed	0	0	0	0	1

Table 1: Summary of primary cultures and resulting cell line. Only *tub-Gal80TS; repo-Gal4; UAS-Ras^{V12}* developed into a line. *attP is an insertion site for transgenes using RMCE


repo-Gal4;UAS-Ras^{V12} RMCE
Days Between Passages
30
25
20
15
10
5
0
P1
P3
P5
P7
P9
P11
P13
P15
P17
P19
P21
P23
P25
P27
P29
Repo RR 1
Repo RR 2
Repo RR 3
Repo RR 4
Repo RR 5
Repo RR 6


repo-Gal4; UAS-wts^{dsRNA}
Days Between Passages
60
50
40
30
20
10
0
P1
P3
P5
P7
P9
P11
P13
P15
P17
P19
P21
P23
P25
P27
P29
Repo 12
Repo 15
Repo 16
Repo 24
Repo 27
Repo 28


repo-Gal4 ; UAS-Ras^{V12} ;UAS-wts^{dsRNA}
Days Between Passages
30
25
20
15
10
5
0
P1
P3
P5
P7
P9
P11
P13
P15
P17
P19
P21
P23
P25
P27
P29
Repo 9
Repo 17
Repo 22
Repo 23
Repo 26

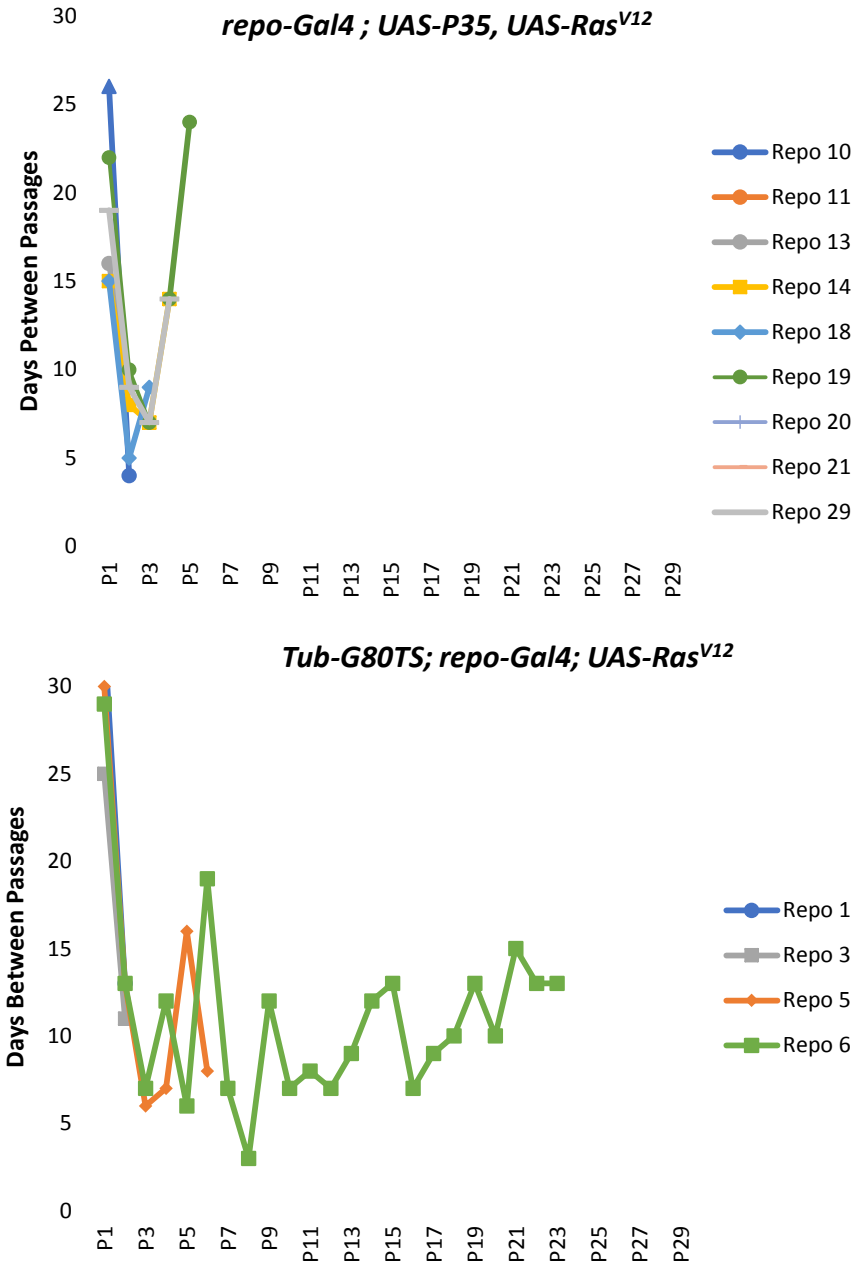


Figure 8: Cell culture passage charts illustrating the number of days between passages. Notably, the days between passages of *repo-Gal4; UAS-Ras*^{V12} ; *UAS-wts*^{dsRNA} are significantly shorter than the other cultures. Only *tub-Gal80TS; repo-Gal4; UAS-Ras*^{V12} developed into a line, the only cell culture that was passaged over ten times besides the *repowts15*, which stained negative for r*epo*, and is not glial in nature. *Note: The vertical axis of the repo-Gal4 > UAS wts*^{dsRNA} *growth chart is scaled differently from the other graphs.*

Characterization of RepoG80-6

To determine if RepoG80-6 had characteristics of glial cells, I tested for expression of *repo* using anti-*repo* antibody (Alfonso and Jones 2002) and a variety of neuronal markers. *22C10* is an antibody that recognizes *Futsch*, a protein necessary for dendritic and axonal growth (Fujita et al. 1982; Hummel et al. 2000). Antibodies that are specific to *HRP* bind to neuronal membranes, inducing membranes of sensory and peripheral nerves, and serve as a neuronal marker (Jan and Jan 1982). Anti-*Fas II* antibodies also are specific to neuronal cells. *Fas II* is a protein that is responsible for the adherence of growing axons to one another and for the process of presynaptic phenotypic plasticity (García-Alonso et al. 1995). The results of the staining experiments are shown in Figure 9. RepoG80-6 expressed *repo* and a small fraction of cells expressed *Fas II*; most cells are glial in nature, but there may be other cell types present in the culture. The existence of cells expressing *Fas II* may indicate the presence of motor neurons, but some studies speculate that anti-*FasII* antibodies may stain cells that are glial in nature (Hebbar and Fernandes 2005). Because the RepoG80-6 cells were negative for anti-*HRP* and *22C10*, other pan-neuronal antibodies, it is plausible that *FasII* is expressed in cells other than neurons, such as a small subset of glial cells.

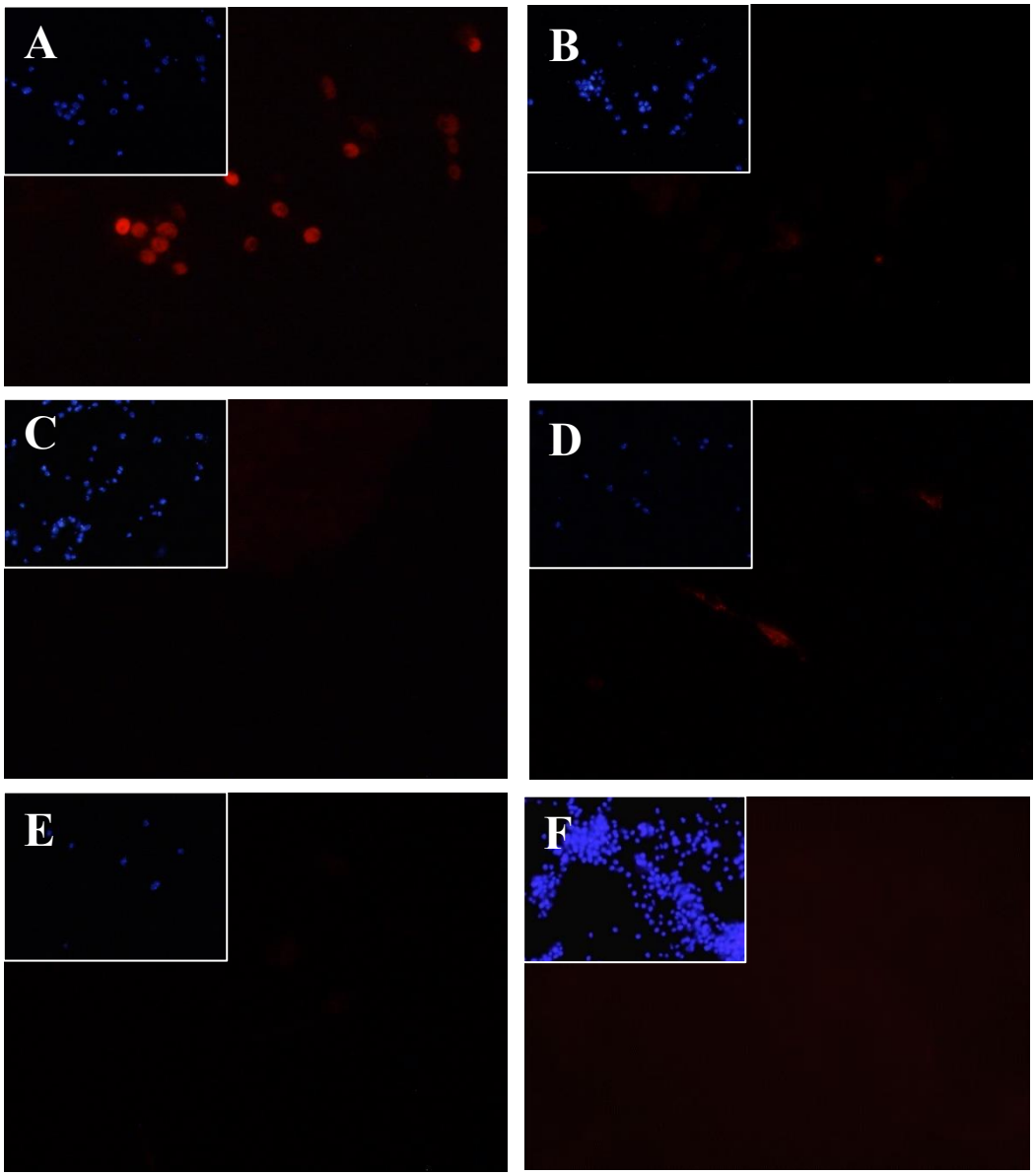


Figure 9: Immunostaining of *Repo*G80-6. *RepoG80-6* expresses *repo* (A) but not *22C10* (B), *HRP* (C). Some cells express *Fas II* (D). E depicts a control in which no primary antibody was added. F shows *repo* antibody staining of an epithelial line.

Karyotype analysis was conducted on RepoG80-6 and the results are shown in Figure 10. Similar to RepoG80-6, human glioblastoma cell cultures sometimes exhibit an extra number of chromosomes (Westphal et al. 1994).

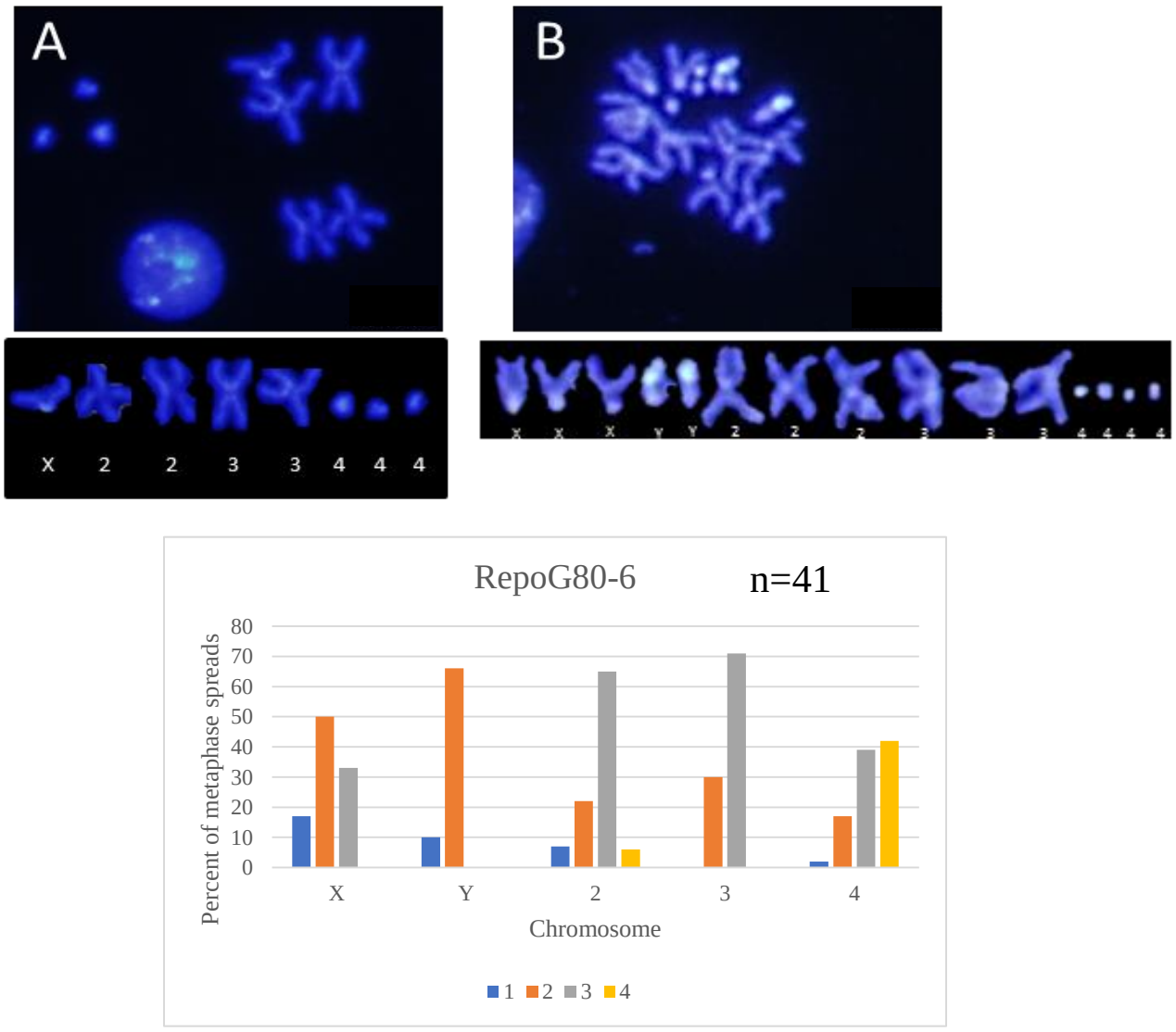


Figure 10: Karyotype of *repo* G80 6 cell line. A,B: Examples of karyotypes found in the RepoG80-6 cell line.

Appearance of another cell type in the RepoG80-6 cell line

After approximately 20 passages, the morphology of the RepoG80-6 cells began to change (Figure 11). Cells appeared to be smaller, with shorter projections (Figure 11). After staining later passages of RepoG806, it was found that the cells were no longer expressing *repo* and were expressing *Fas II*, indicating that the cell type was most likely neuronal in nature (Figure 12). An earlier passage of RepoG80-6 was then thawed and used, which consisted of the glial cell-type.

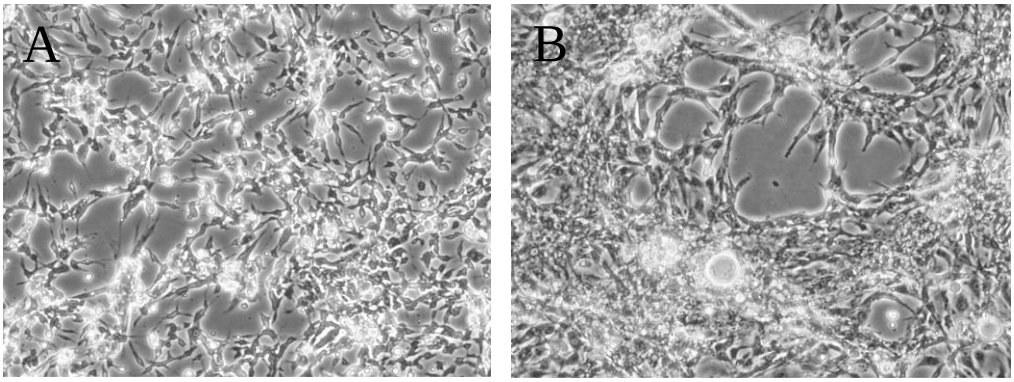


Figure 11: Morphological changes in RG80-6 line. A: RG80-6 no longer had the same morphology at a later passage. B: RG80-6 glial cell-type morphology.

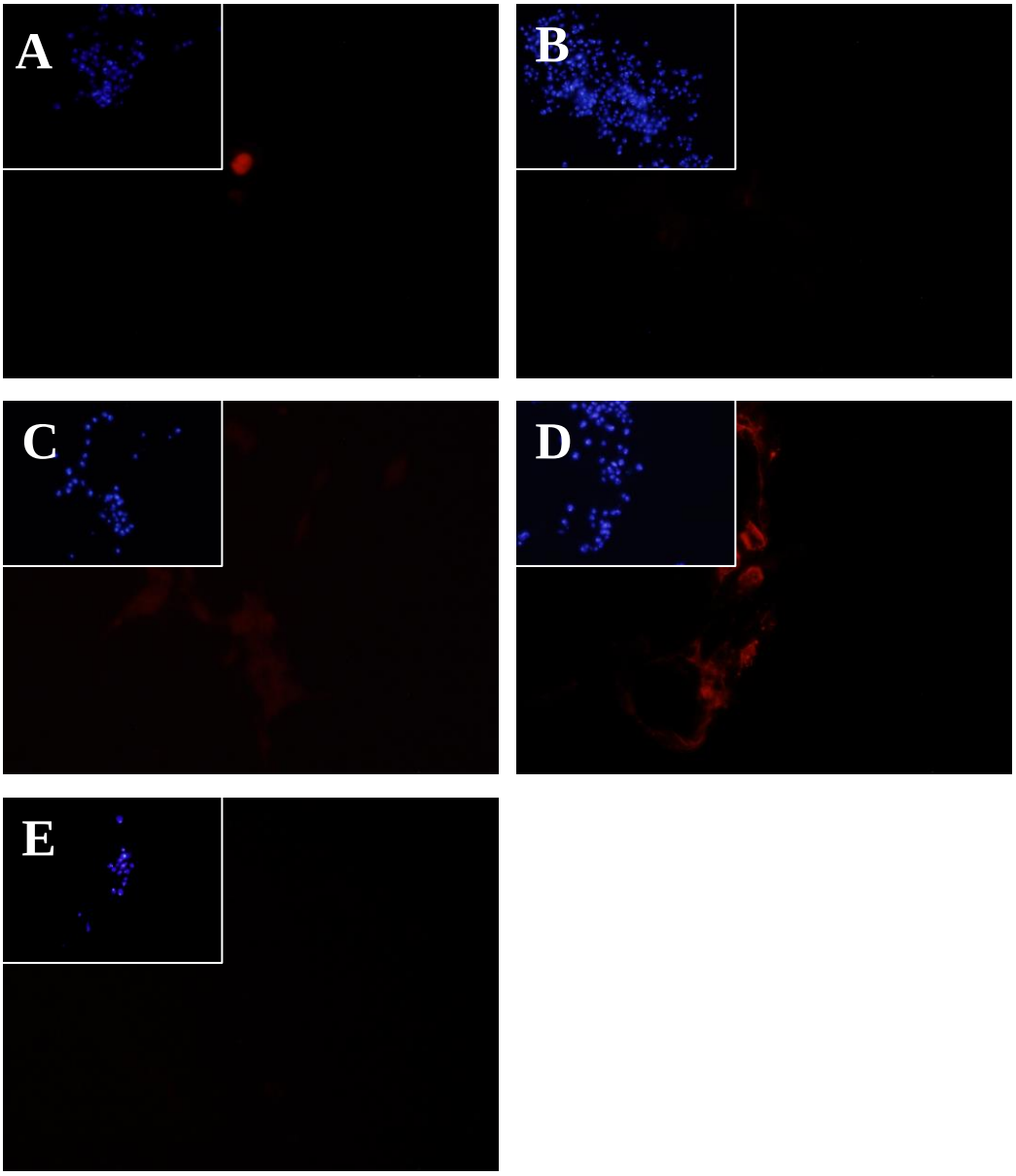


Figure 12: Immunostaining confirms that the cells of RepoG806 changed in morphology and cell type. Cells no longer expressed *repo* (A). The cells still did not express *22C10* (B) or *HRP* (C). They were found to express *Fas II* (D). The cells proliferating in the culture may be a subset of neuronal cells. No primary antibody was added in the control (E).

Conclusion

Because there is a low recovery of glial-specific cell lines, additional spontaneous genetic changes that combine with (or modify) Ras^{V12} expression are likely to be required for glial-specific cell line generation. Despite these genetic changes, the line that I developed is a useful tool that can be used to study glial cells and glial diseases in *Drosophila* and mammals.

FUTURE DIRECTIONS

Using repo-Gal4 as a driver for other tumor suppressor knockdowns in combination with Ras^{V12} expression

The tumor suppressor *brat* is important in the Drosophila brain, promoting asymmetric cell division and directing neural differentiation. *brat* loss of function results in a massively enlarged brain (Chen et al. 2014). TRIM3, the human ortholog of *brat*, also regulates asymmetric cell division. In humans, TRIM3 expression is reduced in samples of glioblastoma (Chen et al. 2014). To gain insights into the functions of TRIM3 in GBM, I utilized the RNAi knockdown of brat to develop a cell line to model GBM. *Repo-Gal4; UAS* $brat^{dsRNA}$ primary cultures have been generated, and more cultures are currently in progress.

I have also developed a fly line that contains transgenes encoding both *brat* ^{dsRNA} and Ras^{V12}. The generation of the stock is shown in Figure 13. The primary cultures are currently being generated.

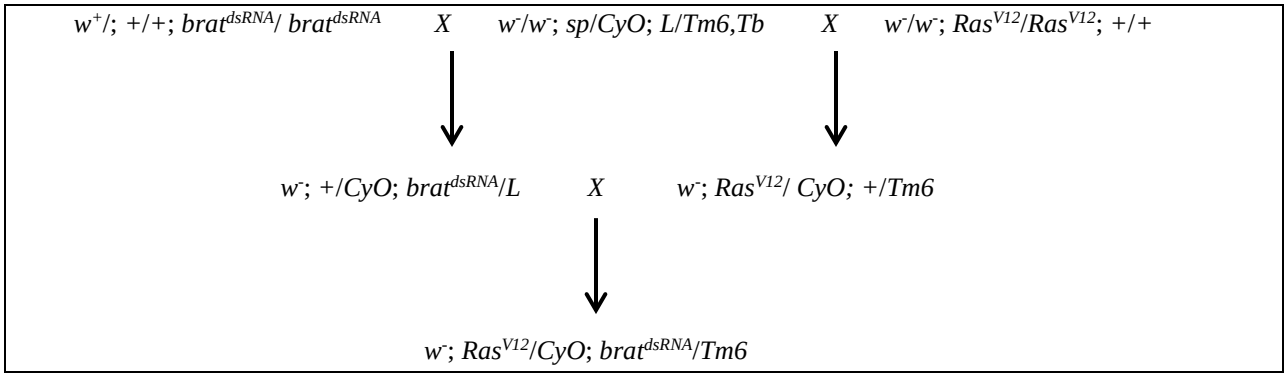


Figure 13: Generation of *UAS-Ras^{V12}; UAS-brat^{dsRNA}* stock.

Using other glial-specific drivers to generate cell lines

I will use *Gal4* drivers for more specific subtypes of glial cells, like the astrocyte, to develop more specific cell lines. To develop an astrocyte-specific fly cell line, I will use *alrm* (*astrocytic leucine-rich repeat molecule)-Gal4*, a *Gal4* driver line expressed only in astrocytes in the antennal lobe of *Drosophila* (Freeman 2015). By crossing an *alrm-Gal4* line with the set of responder lines that I have discussed above, I will be able to generate an astrocyte-specific cell line. Primary cultures of *alrm-Gal4; UAS-Ras^{V12} RMCE* have been established (Figure 14) and are being processed.

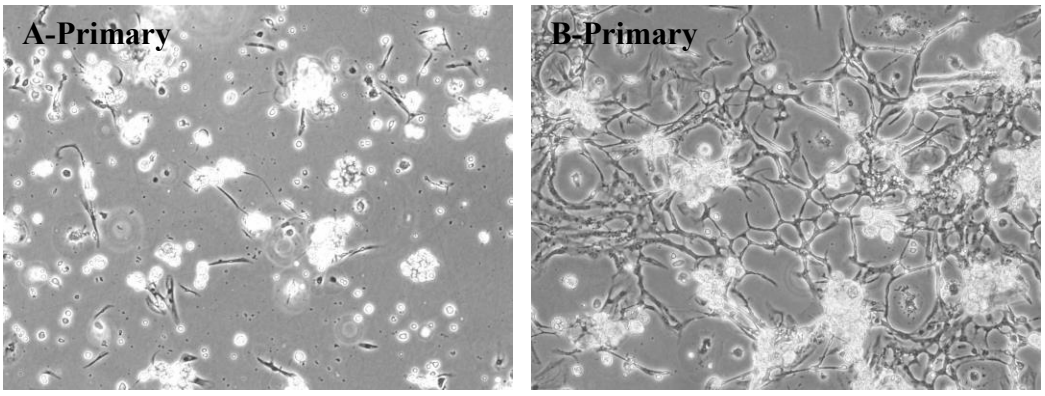


Figure 14: *alrm-Gal4 > UAS-Ras^{V12} RMCE*. Primary cultures on (A) day 10 and (B) day 39.

Further characterization and maintenence of RepoG80-6

I will further characterize the glial-specific cell lines by doing more extensive antibody stains. I will stain for a variety of glial cell markers in RepoG80-6 to find the subtype of glial cells proliferating in culture.

To maintain the RepoG80-6 cell line, I have frozen cells at different passage numbers before the appearance of another cell type. These frozen cells can be thawed and used for further characterization and experiments. Once thawed, another cell type may appear in later passages. It is important that I freeze cells at earlier passages so that there is a supply of RepoG80-6 glial cells. To purify the glial cell line, I am considering cloning cells from a culture of RepoG80-6. Clonal lines are typically more homogenous, so other cell types may not appear (Cherbas and Gong 2014).

RNA Sequencing of RepoG806

RNA sequencing could reveal the gene expression of the cells and highlight certain genes or pathways that may have an important role in the growth of a brain tumor in *Drosophila*. These genes or pathways may have a mammalian equivalent that plays an important role in glioblastoma formation.

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